The demonstration of the existence of regulatory CD8+ T cells in mice that recovered from the first induction of EAE will lead to the study of the specificity of these CD8+ T cells, with regard to both their cellular and molecular targets, as well as to their mechanism of function.

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G Protein Activation of a Hormone-Stimulated Phosphatase in Human Tumor Cells

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The growth-inhibiting peptide hormone somatostatin stimulates phosphotyrosine phosphatase activity in the human pancreatic cell line MIA PaCa-2. This hormonal activation was mediated by a pertussis toxin–sensitive guanosine 5′-triphosphate–binding protein (G protein) in the membranes of these cells. Activation of this G protein by somatostatin stimulated the dephosphorylation of exogenous epidermal growth factor receptor prepared from A-431 cells in vitro. This pathway may mediate the antineoplastic action of somatostatin in these cells and in human tumors and could represent a general mechanism of G protein coupling that is utilized by normal cells in the hormonal control of cell growth.

Pertussis toxin–sensitive G proteins mediate a wide range of functions within the cell, including the control of cell growth (*1*, *2*). Constitutively active mutants of these proteins have been implicated in the pathogenesis of some human neoplasms (*3*). However, the effectors that G protein pathways activate to mediate growth regulation have not been fully identified; these effectors may include phosphotyrosine phosphatases (PTPs). Inhibition of endogenous PTP activity in nontransformed cells in culture can induce cellular transformation (*4*), and the injection of exogenous PTP into *Xenopus* oocytes can reverse the mitogenic effect of insulin-stimulated tyrosine kinase activity (*5*). These findings suggest that PTP activity may counteract the actions of tyrosine kinases to promote normal cell growth, and that interfering with this activity may lead to malignant transformation.

In vitro, the enzymatic activity of PTPs far exceeds that of tyrosine kinases (*6*, *7*). Therefore, PTPs are probably regulated in vivo to maintain a balance between phosphorylation and dephosphorylation. Cellular responses to increased tyrosine kinase activity may generate rapid stimulation of specific PTPs followed by a return to resting levels. G proteins provide hormone receptors with the ability to generate rapid stimulation and termination of effector pathways.

To explore the possibility that G proteins regulate PTP activity, we examined the effects of somatostatin on the undifferentiated human pancreatic cancer cell line MIA PaCa-2 (*8*). This cell line expresses somatostatin receptors and displays hormonally regulated PTP activity (*9*). To characterize the regulation of this PTP activity, we evaluated the action of somatostatin on cell membranes and on whole cells. We measured phosphatase activity with the synthetic substrate *p*-nitrophenyl phosphate (*p*-Npp) (*10*), which releases a spectrophotometrically detectable cleavage product after incubation with phosphatases.

The MIA PaCa-2 cell may contain membrane-associated and cytoplasmic PTPs as well as serine-threonine phosphatase activity. To ensure that the assay was specific for membrane-associated PTP activity, we incubated MIA PaCa-2 membranes with the substrate *p*-Npp and the phosphatase inhibitors microcystin–leucine-arginine (M-LR) and $ZnCl_2$. M-LR is a specific inhibitor of the major serine-threonine protein phosphatases 1 and 2A (*11*). At micromolar concentrations, $ZnCl_2$ inhibits cytosolic PTPs (*12*) more effectively than membrane PTPs. These inhibitors only slightly reduced the basal phosphatase activity (less than 10%), which suggests that a low amount of serine-threonine phosphatase activity was present in our membrane preparations (*13*). The phosphatase activity that remained after inhibition by M-LR was unable to dephosphorylate specific substrates of serine-threonine phosphatases (*14*) and was completely blocked by vanadate (50 μM), an inhibitor of PTP (*15*) (Fig. 1). Therefore, only PTPs were active under the conditions of our assay.

Under conditions of substrate excess, the phosphatase activity from unstimulated membranes was proportional to the length of time and the quantity of membrane proteins assayed (*13*). Inclusion of somatostatin-14 (1 μM) doubled this activity at all times during a 1-hour assay. Vanadate completely blocked both the stimulated and the basal phosphatase activity (Fig. 1). Somatostatin was active over a wide range of concentrations; half-maximal stimulation of phosphatase activity occurred with 2 nM somatostatin. This is similar to the somatostatin concentration that produces a half-maximal effect on growth in this cell line (*16*).

We evaluated phosphatase activity in membranes incubated in the presence of guanosine 5′-triphosphate (GTP) (200 μM) and 5′-guanylylimidodiphosphate (GppNHp, 200 μM), with and without somatostatin-14 (1 μM). GppNHp and GTP (*17*) increased phosphatase activity of MIA PaCa-2 membranes (Fig. 2); the addition of somatostatin further increased this activity (Fig. 2). However, when we incubated cell membranes with the nonhydrolyzable guanine nucleotide analog guanosine 5′-*O*-(2-thiodiphosphate) (GDP-β-S, 200 μM), somatostatin stimulation of phosphatase activity was completely blocked (Fig. 2). This demonstrates that the effect

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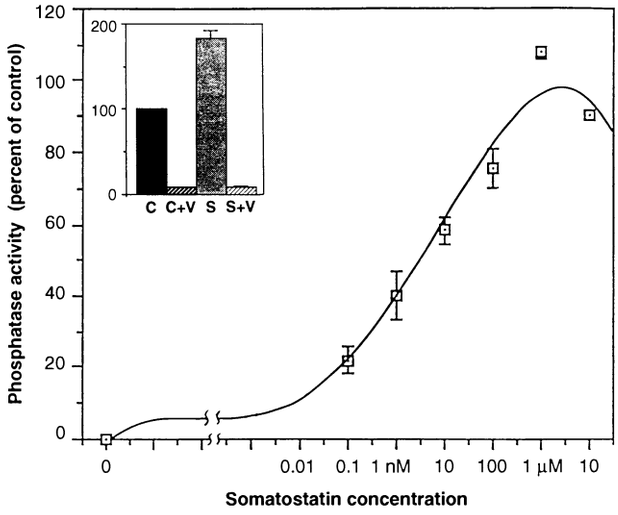

Fig. 1. Dose dependence of stimulation of phosphatase activity by somatostatin. Phosphatase activity was measured as described (*10*) in the presence of various concentrations of somatostatin-14 at 30°C for 10 min. The phosphatase activity is expressed as percent increase over control. Each point represents the mean of separate experimental determinations ± SE (n = 2). Insert: maximal stimulation of phosphatase activity in MIA PaCa-2 membrane proteins by somatostatin and its inhibition by vanadate. Lane 1, membranes alone (C); lane 2, membranes plus vanadate (C + V) (50 μM); lane 3, membranes with somatostatin (S) (1 μM); lane 4, membranes with somatostatin plus vanadate (S + V). Phosphatase activity is presented as percent of control. Each point represents the mean ± SE (n = 4).

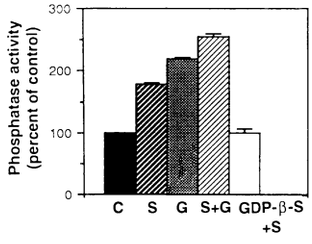

Fig. 2. Effect of nonhydrolyzable GTP analogs on activation of phosphatase activity in MIA PaCa-2 membranes by somatostatin. MIA PaCa-2 membrane proteins (20 μg) were incubated alone (C), with somatostatin-14 (1 μM) (S), with GppNHp (0.2 mM) (G), with both (S + G), or with somatostatin and GDP-β-S (GDP-β-S + S), in the presence of 2.5 mM $MgCl_2$. Phosphatase activity was measured as described (*10*) and is shown as percent of control. Each point represents the mean of duplicate determinations ± SE (n = 3). Statistically significant differences ($P < 0.05$) were determined between groups represented by lanes 1 through 4 with the Scheffe F test.

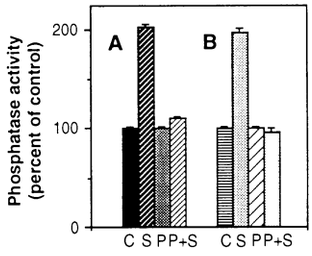

Fig. 3. Effect of pertussis toxin on phosphatase activity. MIA PaCa-2 cells (5 × 10^5 per 10-cm plate) were maintained in fetal calf serum (10%) for 5 days to reach 80 to 90% confluency. Cells were treated with pertussis toxin (120 ng/ml) (Sigma) for 18 hours and then washed and maintained in DMEM (Dulbecco's minimum essential medium). (**A**) Membrane proteins (20 μg) from untreated cells were incubated with buffer (C) or somatostatin-14 (1 μM) (S); membrane proteins from PTX-treated cells were incubated with buffer (P) or somatostatin (P + S) and assayed for PTP activity at 30°C for 10 min. (**B**) PTX-treated or untreated cells were incubated with or without somatostatin (1 μM) in serum-free DMEM at 37°C for 60 min. Membrane proteins (20 μg) from untreated cells (C), cells treated with somatostatin (S), PTX (P), or PTX plus somatostatin (P + S), were incubated in buffer with p-Npp at 30°C for 10 min. Phosphatase activity is presented as percent of control. Points represent the mean of duplicate determinations ± SE (n = 5).

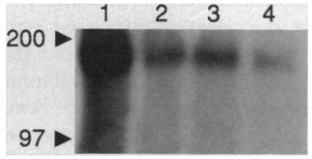

Fig. 4. Dephosphorylation of EGFR prepared from A-431 cells by phosphatases from MIA PaCa-2 membranes. A-431 membrane vesicles enriched for the EGFR were prepared as described (*16*). Membrane vesicle proteins (2 μg) were incubated in 200 μl of kinase buffer [30 mM Hepes (pH 8.0), 15 mM $MnCl_2$, NP-40 (0.75%), and 15 μM ATP] in the presence of EGF (100 nM) and 60 μCi [γ-^{32}P]ATP (5000 Ci/mmol) for 10 min at 37°C. Portions (40 μl) were immediately added to four separate reactions containing MIA PaCa-2 membranes (20 μg) alone (lane 1) or membranes in the presence of 200 μM GppNHp (lane 2), 1 μM somatostatin (lane 3), or 200 μM GppNHp and 1 μM somatostatin (lane 4). Reactions were incubated at 30°C for 30 min in a final volume of 60 μl, which included 50 mM Hepes (pH 7.2), 1 mM EDTA, 5 mM DTT, 20 nM M-LR, 10 μM $ZnCl_2$, 2.5 mM $MgCl_2$, and 0.8 mM ATP, and the reaction was terminated by the addition of 5× Laemmli buffer (12 μl). The samples were separated by SDS–polyacrylamide gel electrophoresis (7% gel) and detected by autoradiography. Relative densities of bands were: Lane 1, 1.00; lane 2, 0.15; lane 3, 0.16; and lane 4, 0.08. Molecular sizes are shown at the left (in kilodaltons).

of somatostatin on phosphatase activity in membranes is mediated by a GTP-binding protein.

We also examined stimulation by somatostatin of phosphatase activity in whole cells. The result was similar in magnitude to the effect seen in membranes (Fig. 3) (*17*). This suggests that both whole cells and membrane preparations contain the accessory proteins necessary for this stimulation. Both effects were completely blocked by pretreatment of the cells with pertussis toxin (PTX) (Fig. 3). Pretreatment with PTX alone did not inhibit the basal phosphatase activity of these membranes, which demonstrates that only the hormone-sensitive activity is inhibited by PTX.

To investigate whether somatostatin-stimulated phosphatase activity could dephosphorylate a physiologically relevant substrate, we prepared an enriched source of epidermal growth factor receptors (EGFRs) from membrane vesicles of the cell line A-431 (*18*). EGFR is selectively phosphorylated in vitro on tyrosine residues and is a substrate for membrane-associated tyrosine phosphatases (*18*). We incubated membrane vesicles from A-431 cells with EGF to activate the intrinsic tyrosine kinase activity of the EGFR. The major phosphoprotein was the 170-kD autophosphorylated EGFR (*13*). Incubation of ^{32}P-labeled membrane vesicles with MIA PaCa-2 membranes in the presence of somatostatin alone, or with GppNHp alone, reduced the intensity of labeled ^{32}P to within the 170-kD band; incubation with somatostatin and GppNHp further reduced the intensity of this band. No effect was seen when the vesicles were incubated with MIA PaCa-2 membranes in the absence of somatostatin or of GppNHp (Fig. 4). Somatostatin did not stimulate dephosphorylation in the presence of GDP-β-S (*13*). This demonstrates that dephosphorylation of exogenous EGFR is stimulated by somatostatin in MIA PaCa-2 membranes by means of a G protein pathway.

Activation of membrane phosphatases by PTX-sensitive G proteins may be a general mechanism for the hormonal control of cell growth. Activation of tyrosine kinase activity after stimulation of G protein–coupled receptors may provide additional hormonal control of cell growth (*19, 20*). Recent reports suggesting that G proteins are also involved in receptor tyrosine kinase signaling pathways (*21, 22*) indicate that the G proteins and the tyrosine kinase–phosphatase signaling pathway may be interconnected at multiple levels.

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Heteromeric NMDA Receptors: Molecular and Functional Distinction of Subtypes

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The *N*-methyl D-aspartate (NMDA) receptor subtype of glutamate-gated ion channels possesses high calcium permeability and unique voltage-dependent sensitivity to magnesium and is modulated by glycine. Molecular cloning identified three complementary DNA species of rat brain, encoding NMDA receptor subunits NMDAR2A (NR2A), NR2B, and NR2C, which are 55 to 70% identical in sequence. These are structurally related, with less than 20% sequence identity, to other excitatory amino acid receptor subunits, including the NMDA receptor subunit NMDAR1 (NR1). Upon expression in cultured cells, the new subunits yielded prominent, typical glutamate- and NMDA-activated currents only when they were in heteromeric configurations with NR1. NR1-NR2A and NR1-NR2C channels differed in gating behavior and magnesium sensitivity. Such heteromeric NMDA receptor subtypes may exist in neurons, since NR1 messenger RNA is synthesized throughout the mature rat brain, while NR2 messenger RNA show a differential distribution.

The excitatory neurotransmitter glutamate can evoke Ca^{2+} influx in neurons of the central nervous system (CNS). This Ca^{2+} influx is critical for activity-dependent synaptic plasticity (*1*) and, if excessive, can lead to neuronal death (*2*). Glutamate-activated Ca^{2+} currents are mediated by the NMDA receptor, a subtype of ionotropic excitatory amino acid (EAA) receptors with distinct pharmacological (*3*) and electrophysiological (*4–7*) features. A key step in characterizing the molecular makeup of this receptor has been achieved (*8*) by the expression cloning of one of its subunits, NMDAR1 (NR1). This study demonstrated that characteristic NMDA receptor properties can reside in homo-oligomeric structures. However, the current amplitudes obtained with NR1 in the *Xenopus* oocyte expression system were low (*8*), a result that predicts that natural NMDA receptors occur in hetero-oligomeric configurations, like other ligand-gated ion channels (*9*).

The primary structure of NR1 (*8*) revealed a family relation to the previously characterized ionotropic EAA receptor subunits (*10, 11*), with which NR1 shares several small sequence islands, particularly in regions around putative transmembrane (TM) segments. By polymerase chain reaction (PCR) amplification of rat brain cDNA with oligonucleotides constructed to detect such conserved sequences (*12*), we found three cDNAs encoding new glutamate receptor subunits, termed NMDAR2A (NR2A), NR2B, and NR2C (Fig. 1). The predicted proteins are between 55% (NR2A and NR2C) and 70% (NR2A and NR2B) identical but are only about 20% identical to homologous subunits (*10, 11*), including NR1 (*8*).

The new subunits, and NR1, carry an asparagine residue in the putative channel forming region TMII, whereas a glutamine or arginine residue resides in the homologous position of the α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA)–selective glutamate receptor subunits (*10*). The exchange by site-directed mutagenesis of either of the latter amino acids for asparagine generates channels characterized by high Ca^{2+}/Cs^{+} and Ca^{2+}/Mg^{2+} permeability ratios and by near linear current-voltage (*I-V*) relations (*13*). Thus, this particular asparagine residue may constitute a distinctive functional determinant in subunits belonging to the NMDA receptor.

When compared to other subunits of the ionotropic glutamate receptor family, two of the three new subunits, NR2A and NR2B, are uniquely endowed with COOH-terminal extensions of greater than 600 residues that contain scattered regions of conserved sequence between the two forms (Fig. 1). The size of these COOH-termini is larger than the extracellular NH_2-terminal segment preceding the first TM region. This finding may cast doubt on the currently postulated topology for ionotropic EAA receptors (*10, 11*), which predicts an extracellular location for sequences distal to the last membrane-spanning region but has not been experimentally verified. If actually located inside the cell, the COOH-terminal sequences, particularly those of NR2A

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